Enterobacteriaceae and *Salmonella* recovered from nonsanitized and sanitized broiler hatching eggs[1]

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Primary Audience: Researchers, Hatchery Managers, Quality Control Personnel

SUMMARY

Sanitizing hatching eggs may reduce the chances that a broiler flock will become colonized with *Salmonella* and reduce the numbers of other microorganisms, such as *Enterobacteriaceae*, that can depress hatchability. An experiment was conducted to determine if a quaternary-biguanide sanitizer applied as foam or spray would reduce *Enterobacteriaceae* or *Salmonella* naturally occurring on broiler hatching eggs. The sanitizer was applied to buggies of 5,040 eggs the day before set (one buggy/treatment at each of 2 settings). Treated eggs were compared with untreated controls. Foam application lowered *Enterobacteriaceae* prevalence at set (0 vs. 18%) and transfer (5 vs. 28%); spraying was effective only when eggs were set (2.5 vs. 11%). At transfer spray, treated and control eggs were 19% *Enterobacteriaceae*-positive. Five *Salmonella*-positives were recorded during the study. No indication that the sanitizer was effective in reducing *Salmonella* prevalence when applied as foam was observed (3/120 vs. 1/120). No *Salmonella* were recovered from spray-treated eggs. No statistically significant difference for *Salmonella* prevalence was noted, but with such a low rate of recovery it is difficult to draw a firm conclusion. However, the sanitizer applied as foam was effective at decreasing the prevalence of *Enterobacteriaceae* (a family of bacteria that includes *Salmonella* and *Escherichia coli*), and is present more often and in higher numbers than *Salmonella*.

Key words: hatchery, microbiology, eggs, sanitation, sanitizer

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DESCRIPTION OF PROBLEM

Salmonella may contaminate fertile hatching eggs [1–3]. Intestinal microflora is less developed in newly hatched chicks than in older birds, making them more susceptible to colonization by *Salmonella* and at lower challenge levels [1]. Therefore, *Salmonella*-positive eggs and embryos can contribute to cross-contamination during incubation and hatching [4]. *Salmonella* contamination that occurs during hatching has been tracked to broiler carcass contamination [5–8]. Sanitizing hatching eggs can decrease *Salmonella* prevalence, reducing a source of

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flock colonization and decreasing the chances of product contamination during processing [9]. *Enterobacteriaceae* is a family of bacteria, which includes *Salmonella,* and may be enumerated to determine microbial quality of eggs. Higher numbers of *Enterobacteriaceae* are an indication of poor sanitation, hygiene, or egg microbial quality [10]. Reducing numbers of *Enterobacteriaceae*, or other populations of fecal origin, can improve hatchability of dirty or floor eggs [11, 12].

Many sanitizers and sanitizing treatments have been demonstrated to be effective in reducing microbial numbers or prevalence [13–19]. Method of application has an effect on the efficacy of chemical sanitizers [16, 20]. Immersion is an effective way to treat eggs with a sanitizer, but is not always the most practical [16]. Application of a sanitizer by foam or spray is less labor intensive than immersion but may result in decreased amount of sanitizer on the egg [20].

In laboratory work with inoculated eggs, *Salmonella* prevalence was decreased when a quaternary-biguanide sanitizer was used [20]. The objective of the current study was to determine the efficacy of a quaternary-biguanide sanitizer (1,200 ppm), applied as foam or spray, in reducing naturally occurring *Enterobacteriaceae* or *Salmonella* from broiler hatching eggs in a commercial setting. The effect of sampling method was also determined. The chemical was applied by foam or spray; eggs were sampled by rinse or shell-crush methods.

MATERIALS AND METHODS

Experimental Design

The broiler hatching eggs used in the current study had been stored for less than 4 d at 18°C and were sanitized at a commercial hatchery. Four settings or hatches were investigated. In 2 of the settings, treated eggs were sanitized by spraying with 1,200 ppm of $4NR_4$ + BG = $4NR_4$ + $C_2H_7N_5R$ (4 quaternary ammoniums and a polyhexamethylenebiguanide hydrochloride moiety) [21], whereas the other 2 were sanitized by foam application of the compound. In each setting, treated eggs were compared with untreated eggs. One buggy of eggs (5,040 eggs/buggy) was subjected to the same treatment at each of 2 settings. Control eggs were obtained from buggies that were from the same breeder flock and collected on the same days as the treated eggs. For each hatch, 20 eggs were collected randomly from the edge row for each buggy before eggs began incubation and also at transfer (20/treatment per hatch). Using an aseptic technique (a new latex glove for each egg), eggs were placed individually into plastic bags and transported back to the laboratory for microbiological analysis.

Application of Sanitizer Solutions

The compound used was $4NR_4 + BG = 4NR_4 + C_2H_7N_5R$ (4 quaternary ammoniums and a polyhexamethylenebiguanide hydrochloride moiety) [21]; this compound was applied as spray or as foam. Sanitizer solutions were prepared the morning of each sample day. Tap water at the hatchery was used as the solvent for diluting the concentrated sanitizer to the appropriate concentration (1,200 ppm). A pump garden sprayer was used for application; depending on the pressure the compound emerged as either spray or foam. This concentration applied by hand-spraying effectively decreased *Salmonella* from inoculated eggshells in a previous study [20, 22].

Sampling Procedure

On the day of collection, eggshells were sampled by rinsing and by a modified crush procedure described by Berrang et al. [23] and modified by Musgrove et al. [24]. Rinsing was performed by adding 20 mL of 1% buffered peptone water [25] and 1 mL of 10% (wt/vol) powdered milk solution to each bag containing an egg and shaking by hand for 1 min. Powdered milk was added to deactivate residual biguanide sanitizer. After rinsing, each egg was removed from the bag aseptically, cracked, opened, and the internal contents (albumen and yolk) discarded. The eggshell and membrane complex were then gently crushed in a gloved hand (a new glove for each egg) and forced into a sterile 50-mL centrifuge tube containing 20 mL of 1% buffered peptone water, and 1 mL of 10% (wt/vol) powdered milk solution.

Microbiological Analyses

Enterobacteriaceae populations were enumerated by pour plating a 1-mL aliquot of each

sample using violet red bile glucose agar [25] in duplicate. An overlay of violet red bile glucose agar was poured after the original agar was set to facilitate the recovery of injured organisms. Plates were incubated overnight at 37°C for 24 h. Plates with dark purple colonies and haloes of bile salt deposition were considered positive and were counted. Counts were converted to $\log_{10}$ colony-forming units per milliliter of sample.

Salmonella pre-enrichment began by overnight incubation in buffered peptone water at 37°C for 18 to 24 h, selectively enriched by adding 0.1 mL to Rappaport-Vassiliadis [25] and TT (tetrathionate) broth [25] at 42°C for 18 to 24 h, and then plated onto BGS (brilliant green sulfa) agar and XLT4 (xylose lysine tergitol 4) plates [25]. After the plates were incubated at 37°C for 18 to 24 h, presumptive *Salmonella* colonies were selected and used to inoculate triple sugar iron and lysine iron agar slants [25]. Any isolates giving typical reactions after 18 to 24 h at 37°C were confirmed serologically using a commercial polyclonal latex agglutination kit [26]. The confirmed colony was then streaked for isolation onto plate count agar and incubated at 37°C overnight. This procedure was repeated twice to ensure clonality. After being confirmed as *Salmonella,* isolates were serogrouped with commercial antisera [26].

Statistical Analysis

Enterobacteriaceae numbers were lower than could be accurately analyzed. A chi-squared test for independence was conducted on the *Enterobacteriaceae* and *Salmonella* prevalence determined for each sanitizer application method (spray or foam) and sample collection time (before setting or before transfer) [27] for each sampling method (rinse or crush). For all analyses, significance was determined at $P < 0.05$.

RESULTS AND DISCUSSION

Enterobacteriaceae are a family of bacteria that can be used through enumeration or recovery to give a general idea of the level of hygiene. The higher the numbers or more often this population is recovered, the lower the sanitation level and poorer the bacterial quality in terms of cleanliness [10]. Results for *Enterobacteriaceae* prevalence are presented in Table 1. Though each egg sample was enumerated, the *Enterobacteriaceae* counts were so low that the average per treatment group for both control and treated eggshells was below the precision for pour plate techniques [28]. When the quaternary ammonium-sanitizing compound was applied as foam, *Enterobacteriaceae* prevalence was decreased significantly ($P < 0.05$) when compared with untreated controls at set (0 vs. 18%) and transfer (5 vs. 28%). When sprayed, the sanitizing compound was only effective at set (2.5 vs. 11%). At transfer, spray-treated and unsprayed controls averaged 19% *Enterobacteriaceae*-positive.

In the current study, the sanitizer was more effective at reducing *Enterobacteriaceae* prevalence when applied as foam compared with liquid application. Foams are not always the best choice because they cling and are difficult to rinse off. This characteristic is a benefit when used as a sanitizer because the longer the foam clings, the greater the contact time with bacteria present on the eggshell. When sanitizers are applied as foam, it increases the surface area of the compound, allowing more direct contact with microorganisms. This may have contributed to the improved efficacy in our study. Additionally, a liquid will dry more quickly than foam [29, 30]. Patterson et al. [31] demonstrated that foaming with chlorine dioxide was more bactericidal than formaldehyde fumigation for fertile duck eggs, particularly for soiled eggs. Chlorine dioxide foam reduced *Escherichia coli* and *Streptococcus fecalis* numbers by 1.6 and 3.5 $\log_{10}$ cfu/mL, respectively, on inoculated hen eggshells [31]. Method of application has been demonstrated to influence the effectiveness of broiler hatching egg sanitizers [20]. Whereas dips are initially most effective, they are not always practical and sometimes decrease hatchability if the solution is not changed frequently and temperature is not maintained in a commercial application [16, 32]. Provided a means of producing the foam exists, it is easily applied by spraying.

Salmonella prevalence was very low in the current study. In all, *Salmonella* was detected in only 5 samples. A single control sample was positive at set (during a spray treatment). At the second hatch foam transfer, 3 treated samples

Table 1. Recovery ratio of broiler hatching eggshell samples contaminated naturally with *Enterobacteriaceae* that had been sanitized by foam or spray [1,200 ppm $4NR_4$ + BG = $4NR_4$ + $C_2H_7N_5R$ (4 quaternary ammoniums and a polyhexamethylenebiguanide hydrochloride moiety) and untreated controls at set and at transfer]

Treatment[1]	Foam set[2]	Foam transfer	Spray set	Spray transfer
Treat				
Rinse	0/20[3]	0/40	1/40	1/40[a]
Crush	0/20	4/40	1/40	14/40[b]
Control				
Rinse	5/20	12/40	4/40	6/40
Crush	2/20	10/40	5/40	9/40
Total				
Treat	0/40[B]	4/80[B]	2/80[B]	15/80
Control	7/40[A]	22/80[A]	9/80[A]	15/80

[a,b]Rates of recovery (no. positive/total number of samples) for egg samples grouped by treatment and sample type (rinse vs. crush) with different letters are significantly different ($P < 0.05$).

[A,B]Rates of recovery (no. positive/total number of samples) for eggs grouped by treatment with different letters are significantly different ($P < 0.05$).

[1]Eggs were sanitized (Treat) or left untreated (Control) and eggshells were sampled by rinse and crush.

[2]Application method of treatment (foam or spray) and time of sampling (set or transfer).

[3]The study was initiated after the first foam set sample; therefore, half as many samples were made.

and a control sample were all determined to be *Salmonella*-positive. None of the differences between treated and controls were statistically significant. Because the incidence of naturally occurring *Salmonella* may be very low, many studies that determine the efficacy of a sanitizer or sanitizing treatment use *Salmonella*-inoculated eggs [32–35]. The tradeoff is that the numbers used are not reflective of *Salmonella* contamination that occurs naturally. Determining statistical significance in the reduction in prevalence for a microorganism that occurs so rarely requires sampling more eggs than is practical for most laboratories. Confirmations were performed on cultures derived from a single colony. Serogrouping antisera demonstrated that the isolates were group B or C_1. These 2 serogroups are the most common recovered from broilers and many other poultry-related samples [9, 36].

Method of sampling eggshells can affect recovery rates, particularly for those populations that are in low numbers or occur more sporadically [37, 38]. Musgrove [24, 37] determined that sampling a slurry composed of crushed shells and membranes was more effective than rinsing the eggshells for recovery of *Enterobacteriaceae* and *Salmonella*, particularly after the eggs were washed. As in the present study, eggs were first rinsed and then sampled by crushing, as described previously. The rinse method was equally effective for unwashed eggs, particularly when enumerating aerobic microorganisms [24]. In the current study, in terms of *Enterobacteriaceae*, this was true at only 1 sampling point (spray treated eggs at transfer). In a laboratory trial, Spickler et al. [39] found no advantage over the modified crush method compared with rinsing for aerobic bacteria. However, the diluent may have cooled before shell maceration. Kawasaki et al. [38] found that using warm diluent increased recovery 10-fold. Of the 5 *Salmonella* recovered in the current study, 4 of them were recovered from crushed shells and membranes, indicating that a method that includes shells and membranes may be most effective for organisms present sporadically and in low numbers, such as *Salmonella*.

Hatchability was determined for untreated and treated eggs (regardless of application method). The current study was conducted in a commercial facility. Often, less control and sample availability exist when working in commercial facilities compared with the laboratory setting. Hatchability was lower for treated eggs than for untreated controls (78 vs. 85%). In laboratory trials with bench-top incubation, no difference in egg weight or hatchability was observed when control eggs were compared with treated eggs (89 vs. 92%). It is unclear why the treated egg hatch rate was lower than untreated con-

trols in commercial trials. However, after eggs were treated they were left in an egg-holding room that is used for several purposes. It took up to 15 min for eggs to dry after treatment, with sprayed eggs drying more quickly than those that were foam treated. Perhaps if they had been moved into a cleaner environment, hatchability may not have been affected. Mitchell et al. [40] demonstrated that improving air quality can decrease egg contamination in poultry operations, whereas Davies and Wray reported that hygiene affects hatchability as well as *Salmonella* prevalence [41]. Patterson et al. [31] reported increased hatchability of clean and soiled duck eggs that had been foam treated compared with uncleaned soiled eggs. In 2 subsequent laboratory incubation trials (R. Buhr and D. Bourassa, unpublished data), no detectable differences in egg weight loss during incubation to transfer was detected for eggs sprayed with $4NR_4$ + BG (300 ppm 8.5%, 600 ppm 8.8%, or 1,200 ppm 8.6%) compared with unsprayed (8.6%), water-sprayed (9.1%), or hydrogen peroxide-sprayed eggs at 14,000 ppm (8.5%). These results indicate that the lower hatchability percentages in the commercial hatchery are not likely attributable to a potential reduction in egg weight loss of eggs sanitized with $4NR_4$ + BG at 1,200 ppm by foam or spray in the commercial hatchery.

Excess fertile broiler eggs may be sent to breaker plants for use in liquid or dried egg products [42]. Recent FDA regulations may eliminate some of the chances to use these eggs if new temperature holding conditions (<45°F) are not observed [43]. Numbers and prevalence of *Enterobacteriaceae* recovered in the current study are lower than reported for unwashed shell eggs from layers. *Salmonella* prevalence was also lower than is sometimes determined for shell eggs [37]. In the United States, liquid egg products are pasteurized at temperatures that decrease *Salmonella* numbers by 100,000-fold [43]. Based on these results, the microbial quality of these eggs would be suitable for use at breaker facilities.

Regardless of how eggs are used, for hatching or consumption, reducing *Salmonella* requires attention at all stages of production from breeder farm through processing. Sanitizing broiler hatching eggs is one of steps that can be taken to decrease *Salmonella* in poultry flocks. Effective hatchery hygiene and fertile egg sanitation supports the effort in providing safer poultry products to consumers.

CONCLUSIONS AND APPLICATIONS

1. The sanitizing chemical $4NR_4$ + BG (containing 4 quaternary ammoniums and a biguanide) at 1,200 mg/L when applied as a foam was effective at reducing *Enterobacteriaceae* prevalence at set and at transfer of broiler hatching eggs during incubation.
2. The sanitizing chemical $4NR_4$ + BG (containing 4 quaternary ammoniums and a biguanide) at 1,200 mg/L when applied as a spray was effective at reducing *Enterobacteriaceae* prevalence at set but not at transfer of broiler hatching eggs during incubation.
3. *Salmonella* contamination of egg shells was present sporadically. In the current study, no effect on *Salmonella* prevalence due to treatment by either means of application was determined.
4. In our study, rinsing and crushing methods were equally efficient at recovering *Enterobacteriaceae*.

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